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Sulfur Mustard(SM) causes death and detachment of the basal cells of the epidermis, and thus is a strong vesicating agent. To design a therapeutic strategy, we have examined the role of apoptosis in SM vesication. We found that SM induces differentiation and apoptosis in human keratinocytes. Furthermore, apoptosis occurs via both a calmodulin-dependent mitochondrial pathway as well as by a FADD-dependent death receptor pathway. In the last year, we found antiapoptotic members of the Bcl-2 family are down-regulated and the proapoptotic member, Bax, is upregulated. In addition, Bad is dephosphorylated, leading to mitochondrial pathways of apoptosis. These events were inhibited by CaM antisense or CaM chemical inhibitors. Finally, we designed a FADD-DN vector which also inhibits the death receptor pathway of SM-induced kertinocyte apoptosis. The CaM-Bcl-2 and Fas-FADD pathways therefore represent attractive targets for therapeutic intervention.

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1. OVERVIEW

Sulfur Mustard (bis-(2-chloroethyl) sulfide; SM) is a highly reactive compound that induces the death and detachment of the basal cells of the epidermis from the basal lamina. Thus, SM is a strong vesicating agent. In an effort to identify therapeutic targets for intervention of vesication induced by SM, we have been defining the mechanisms for SM toxicity in cell culture, in grafted human epidermis, and in transgenic animal models. In cell culture, we have been examining the pathways in both keratinocytes and in dermal fibroblasts. We found that whereas human dermal fibroblasts may contribute to the vesication response by releasing degradative cytosolic components extracellularly after a PARP-dependent SM-induced necrosis, keratinocytes display markers of an apoptotic death, as well as those of terminal differentiation (Rosenthal et al., 1998; Rosenthal et al., 2001). Furthermore, we found that SM-induced apoptosis in keratinocytes is controlled by both death receptor and mitochondrial pathways (Figure 1).

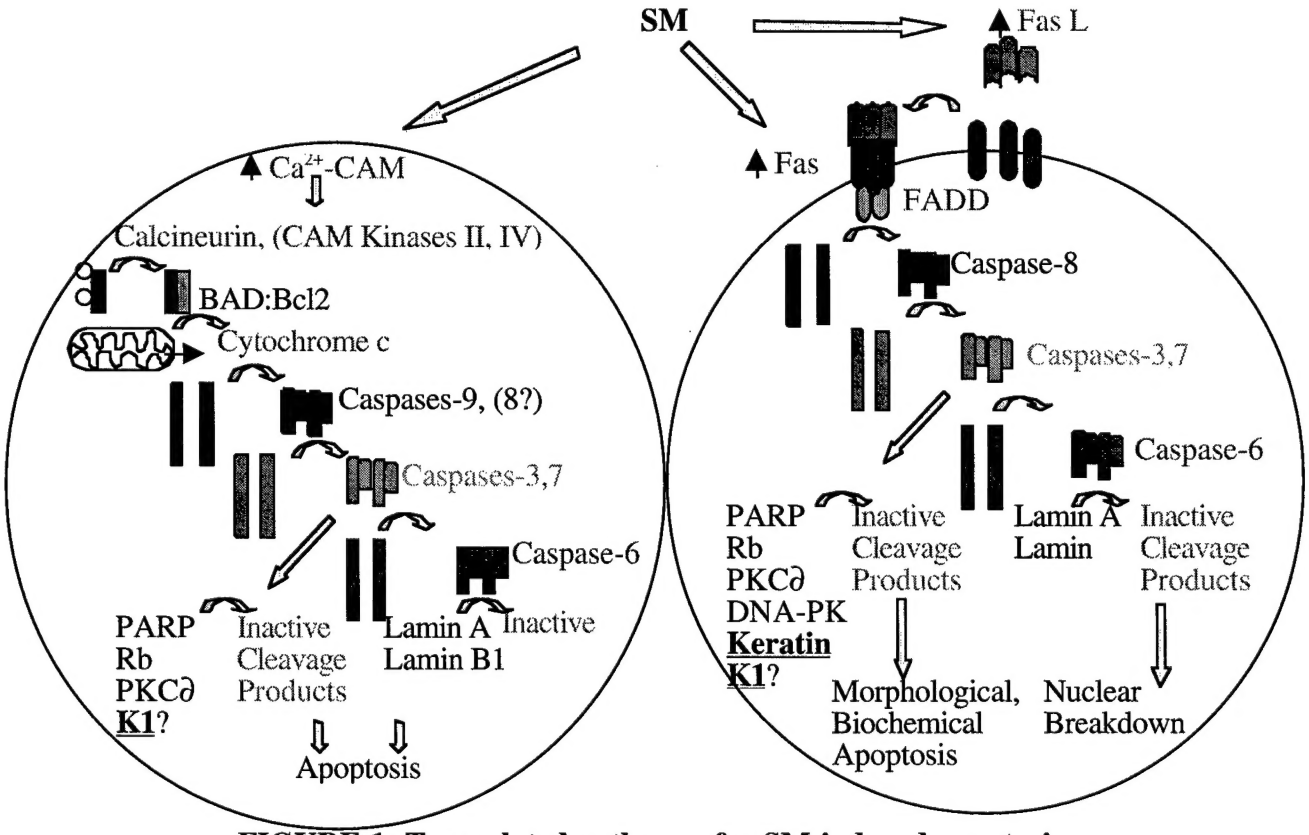


FIGURE 1: Two related pathways for SM-induced apoptosis

Fas pathway

A number of other agents that induce apoptosis have been found to exert their influence by elevating the levels of TNFα or Fas L, or their receptors. Members of the Fas/TNFR family and their ligands may be induced at the level of transcription following stimulation by apoptosis-inducing agents, such as doxorubicin (Friesen et al., 1996; Herr et al., 1997), and p53 has been shown to play a role in the upregulation of Fas receptor (Owen-Schaub et al., 1995). Importantly, p53 is also rapidly upregulated in keratinocytes following SM treatment, and may also play a role in SM-induced apoptosis (Stöppler, et al., 1998). UV has also been shown to activate receptors from the Fas/TNFR family, either by direct crosslinking (Aragane et al., 1998) or by upregulation of the Fas ligand or receptor (Takahashi et al., 1995). Subsequent recruitment of FADD (Chinnaiyan et al., 1995), FLASH (Imai et al., 1999) and caspase-8 (Medema et al., 1997), to the death-inducing signaling complex (Kischkel et al., 1995), induces the activation of caspase-8 (Medema et al., 1997), followed by the activation of the executioner caspases (caspases-3, -6, and -7). In a previous study, it was determined that UV up-regulates the levels of TNFα in keratinocytes (Kock et al., 1990; Leverkus et al., 1998; Schwarz et al., 1995), and that a TNFα-neutralizing antibody affords partial protection from apoptosis (Schwarz et al., 1995). Another study showed that the percentage of keratinocytes expressing Fas receptor increases from 5% to 32% following treatment with IFNγ, corresponding with the percentages of cells susceptible to Fas-mediated apoptosis (Takahashi et al., 1995). FADD protein is common to three different apoptosis-signaling receptors: Fas, TNFR1, and DR3 (Chinnaiyan et al., 1995; Chinnaiyan et al., 1996; Chinnaiyan et al., 1996). In the first year, we provided evidence that SM upregulates both Fas and Fas L in primary human epidermal keratinocytes. When we examined the expression of Fas receptor in primary keratinocytes by Western analysis, the levels were negligible, consistent with previous results. However, following treatment with SM, there was an up-regulation in the levels of both Fas receptor and Fas ligand. We also have observed the activation of markers of apoptosis that are consistent with a Fas ligand-receptor interaction, including caspase-8, caspase-3, and PARP cleavage. In the second year we show that a truncated dominant-negative form of FADD (FADD-DN) blocks SM apoptosis in keratinocytes and may prevent vesication in grafts. Fas-blocking antibody experiments also show that SM may partially exert its apoptotic effect partly via a Fas- FasL interaction. In addition, experiments are currently underway using Fas k/o mice to validate this approach.

Mitochondrial pathway of apoptosis and the roles of CaM and Bcl-2 family members.

In some systems, cytochrome c release from the mitochondria has been shown to be an early event in DNA-damaged cells, prior to caspase activation (Bossy-Wetzel et al., 1998). This pathway involves loss of mitochondrial membrane potential, followed by release of cytochrome c, apoptosis-inducing factor (AIF), and possibly procaspases-2 and –9 (Susin et al., 1999), followed by the activation of caspase-9, in the presence of Apaf-1, and the subsequent activation of caspase-3. An important group of proteins that are involved in the stability of mitochondria and cytochrome c release are members of the Bcl-2 family. The Bcl-2 family is comprised of both pro and anti-apoptotic members. The antiapoptotic members of this family include Bcl-2 and Bcl-xL, while proapoptotic members include Bad, Bax, and Bak. It is believed that multimers of Bcl-2 and Bcl-xL form pores in the mitochondrial cell membrane that prevent depolarization, permeability transition, and ultimate activation of caspase-9. Bad, Bax, and Bak can heterodimerize with Bcl-2 and Bcl-xL, preventing these stabilizing pores from forming, and thus facilitate the release of cytochrome c and activation of caspase-9. Thus the activation of this pathway of apoptosis depends ultimately on the relative levels of the pro and anti apoptotic members of the Bcl-2 family.

Calmodulin (CaM) plays a role in regulating the levels of Bcl-2 family members and thus the mitochondrial pathway of apoptosis. Several members of this family have been shown to interact directly with the CaM-regulated phosphatase calcineurin, which can dephosphorylate (Wang et al., 1999) and regulate the intracellular localization (Shibasaki et al., 1997) and stability (Haldar et al., 1995) of members of the Bcl-2 family. Bcl-2 transcription is also regulated via a CaM-dependent pathway (Gomez et al., 1998). SM in turn has been shown to alter calcium and CaM homeostasis in keratinocytes (Mol and Smith, 1996; Ray et al., 1995; Ray et al., 1993), and calcium-buffering experiments have supported the role of calcium in the etiology of SM-induced cytotoxicity (Ray et al., 1996).

Published studies, including my own, utilizing specific inhibitors of CaM have demonstrated the importance of Ca^{2+} -CaM complexes in programmed cell death (Pan et al., 1996; Sasaki et al., 1996); Rosenthal, et al., 1998). Cyclosporin A-sensitivity of apoptosis in certain systems also suggests a role for Ca^{2+} -CaM complexes in programmed cell death. Cyclosporin binds to a family of cytosolic receptors (cyclophilins); the complex then binds to and suppresses the serine/threonine phosphatase calcineurin, which in turn is regulated by Ca^{2+} -CaM complexes in programmed cell death (Shi et al., 1989). Interestingly, in numerous recent studies, Bad,

a pro-apoptotic member of the Bcl-2 family, has been implicated as a key player in programmed cell death (Datta et al., 1997; del Peso et al., 1997; Hsu et al., 1997; Scheid and Duronio, 1998; Yang et al., 1995; Zha et al., 1997; Zundel and Giaccia, 1998). Calcineurin has been shown to interact with the Bcl-2 family members (Shibasaki et al., 1997), and to dephosphorylate Bad (Wang et al., 1997). This dephosphorylated form of Bad can interact with Bcl-2 or Bcl-X_L and induce apoptosis (Zha et al., 1997).

We have shown that **SM** induces both terminal differentiation and apoptosis in human keratinocytes. Further, we have demonstrated that these processes are Ca^{2+} and CaM dependent, and involve the activation of caspase 3. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to **SM**.

2. TIME COURSE AND DOSE RESPONSE OF BCL2 FAMILY PROTEINS DURING SM-INDUCED APOPTOSIS IN CONTROL AND CALMODULIN-DEPLETED CELLS

2.1 Introduction

During the first year, I focused on the roles of Ca^{2+} /CaM in the modulation of differentiation and apoptosis in epidermal cells, and potentially involved in vesication. I have utilized much of the same technology that I have successfully employed previously to answer an essential question- How does Ca^{2+} /CaM alter the apoptotic and differentiation responses in keratinocytes, and can these pathways be modulated to alter SM vesication in animal models (and ultimately, in humans)?

In the first year, we showed that SM induces both terminal differentiation and apoptosis in human keratinocytes. Further, we have demonstrated that these processes are Ca^{2+} and CaM dependent, and involve the activation of a number of caspases. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to SM. In the second year, we focused on the role of the Bcl-2 family of proteins, which are involved in the mitochondrial pathway of apoptosis, leading to the activation of caspase-9. Higher levels of Bcl-2 and Bcl-xL stabilize the mitochondria, preventing the release of proapoptotic constituents, including caspase-2, caspase-9, apoptosis inducing factor (AIF) and cytochrome c. This may be due to the pore forming ability of these proteins, which prevents mitochondrial permeability

transition. On the other hand, high levels of the proapoptotic family members including Bax, Bak, and Bad favors depolarization of the mitochondria, leading eventually to the activation of caspase-9. Thus we performed an extensive series of time course and dose response experiments to determine the relative levels of the Bcl-2 family members following SM exposure. In addition, we examined the dose-response and time course following exposure of CaM depleted cells to SM. Finally we specifically examined the role of phosphorylation of Bad and its intracellular localization following SM exposure and determined the role of CaM in this process.

2.2 Materials and Methods

(1) Culture of primary and immortalized human keratinocytes, and exposure to SM.

a. Cells. Normal human foreskin keratinocytes (HFK) were either obtained as primary cultures from Clonetics (San Diego, CA), or prepared from human foreskin keratinocytes as described previously, and maintained in serum-free Keratinocyte Serum-Free Medium (SFM). Cells are grown to 80% confluency and split 1:5. HFK in 75 cm2 tissue culture flasks to 60-80% confluency, then exposed to HD diluted in KGM. Media is not changed for the duration of the experiments

b. Chemicals. SM (bis-(2-chloroethyl) sulfide; >98% purity) is obtained from the US Army Edgewood Research, Development and Engineering Center.

(2) Measurement of levels of Bcl-2 family members

a. Antibodies. One of the first tasks was to determine the molecular ordering of events leading to SM-induced apoptosis. We have extensive experience utilizing Western analysis to detect the levels of Bcl-2-related proteins. We first tested a number of antibodies from commercial and collaborative sources for their sensitivities and specificities using cells treated with known apoptosis-inducing agents, such as anti-Fas antibody, as controls. We developed an inventory of excellent antibodies specific for all relevant Bcl-2-related proteins (Table 1). In addition, we obtained antisera that detects the substrate cleavage products for lamin B1, a substrate of caspase-6. Thus, by performing time-course experiments in Aim I, as well as inhibitor studies,

we have been able to begin to determine the sequence of events, as well as the regulatory molecules (such as Bcl-2), involved in SM-induced apoptosis. All antibodies indicated in Table 1 were tested and used successfully in our laboratory.

b. Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, the blots were incubated with monoclonal or polyclonal antibodies (above) and then detected with appropriate peroxidase-labeled secondary antibodies (1:3000 dilution) and enhanced chemiluminescence (ECL, Amersham). Immunoblots were sequentially stripped by incubation for 30 min at 50 °C with a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7), blocked again, and reprobed with additional antibodies to accurately compare different proteins from the same filter. Typically, a filter could be reprobed three times before there was detectable loss of protein from the membrane, which was monitored by Ponceau S staining after stripping.

c. ***Antibodies.***

Table 1

Antibody (kDa)	type(clone)	Source	Dilution (conc.)
Calmodulin (17)	monoclonal (1F11 + 6D4)	Sigma	1:1000
K1 (67)	polyclonal	Babco	1:50
K14 (50)	monoclonal	Sigma	1:200
Involucrin (68)	monoclonal (SY5)	Sigma	1:200
Fibronectin (220; 94)	polyclonal	Sigma	1:500
FADD (24)	monoclonal (1)	Transduction Labs	1:250
AU1	monoclonal (AU1)	Babco	1:1000 (1 μg/ml)
Lamin B1	monoclonal	Calbiochem	1:100 (1 μg/ml)
p53 (53)	monoclonal (pAb421)	Calbiochem	1:200 (0.5 μg/ml)
Bcl-2 (25)	monoclonal (4D-7)	Biomol	1:200 (1 μg/ml)
Bcl-XL	polyclonal	Calbiochem	1:40 (2.5 μg/ml)
Bax (21)	polyclonal	Calbiochem	1:50 (2 μg/ml)
Bad	polyclonal	New England Biolabs	1:1000
Phospho-Bad	polyclonal	New England Biolabs	1:1000
Bax	polyclonal	Trevigen	1:10,000 (0.2 μg/ml)
Bcl-2 (YTH-8C8)	monoclonal	Trevigen	1:500 (2 μg/ml)
Bcl-xL	polyclonal	Santa Cruz	1:200 (1 μg/ml)
Bid	polyclonal	Trevigen	1:1000 (0.5 μg/ml)
Bim	polyclonal	Trevigen	1:1000 (0.5 μg/ml)

2.3 Results

Changes in endogenous levels of Bcl-2 family members during SM-induced apoptosis

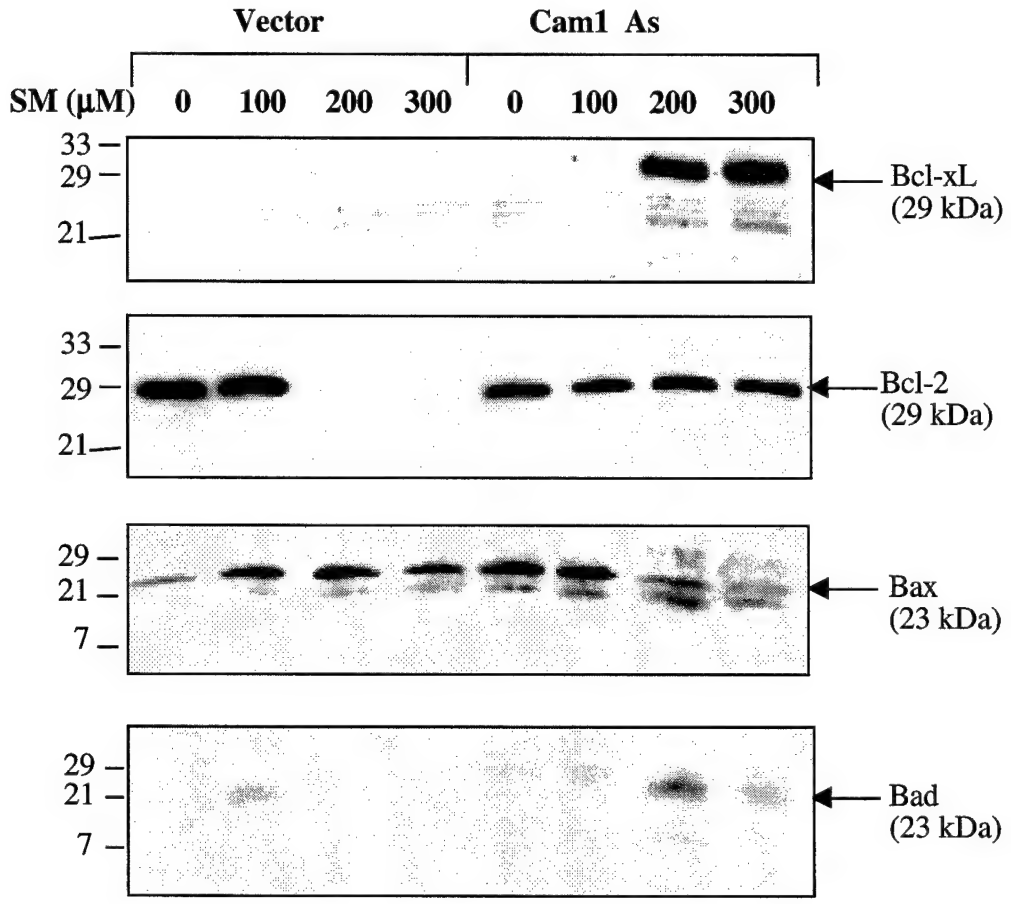


Fig. 2. Dose response for expression of Bcl-2 family members

Some DNA damaging agents, including chemotherapeutic agents, have been shown to alter the levels of Bcl-2-related proteins, resulting in the activation of caspase-9. In addition, overexpression of either Bcl-2 or Bcl-xL can protect against certain apoptosis-inducing agents. We first performed a dose response, in which HFK were exposed to either 0 μM, 100 μM, 200 μM or 300 μM SM, and extracts were subjected to immunoblot analysis. As shown in Fig. 2, very low levels of Bcl-xL are observed in control HFK at all levels of SM tested. However, when CaM levels are reduced by antisense expression, Bcl-xL levels are induced following exposure to vesicating doses of SM (200 μM and 300 μM). Bcl-2 is expressed at higher levels in control HFK, but, levels are significantly reduced in the presence of 200 μM and 300 μM SM. Following CaM depletion by CaM antisense,

Bcl-2 is maintained at high levels following treatment with SM at all doses. We also examined the expression levels of the proapoptotic proteins, Bad and Bax. As seen in Fig. 2, the levels of Bax increase with increasing concentrations of SM in control HFK. However, in CaM-depleted cells, Bax levels are reduced at higher doses of SM. The levels of Bad were generally low in both control and CaM-depleted cells. However since the phosphorylation status of Bad determines its apoptotic activity, we performed further analysis, which is described below.

Since our dose-response experiments suggested that CaM depletion blocks mitochondrial pathways of SM-induced apoptosis via upregulation of Bcl-2 and Bcl-xL, as well as the down regulation of Bax, we performed a series of experiments to determine the time course for the change in the levels of these proteins in response to a vesicating dose (300 μM) of SM both in control and in CaM-depleted HFK.

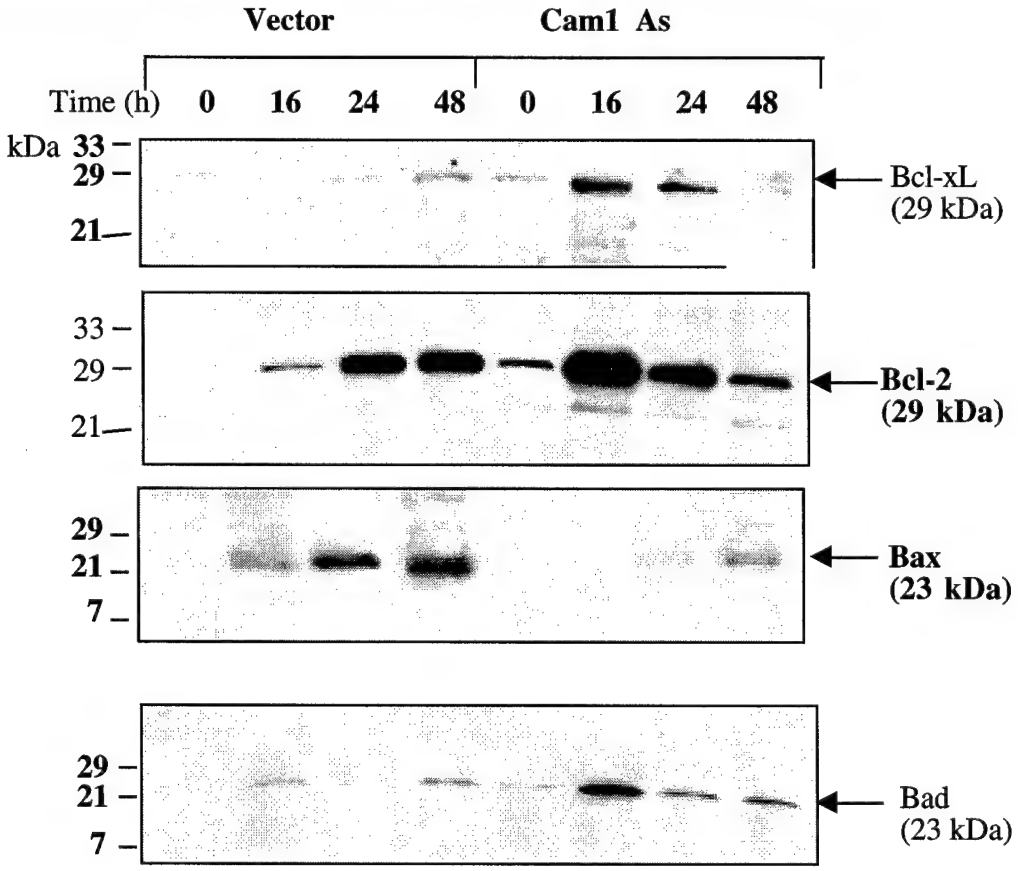


Fig. 3. Time course of Bcl-2 family member expression following SM exposure of control and CaM-depleted HFK.

Fig. 3 shows that the levels of Bcl-2 and Bcl-xL increase more rapidly in CaM-depleted cells and to higher levels following exposure to 300 μM, than control HFK. In contrast, the levels of Bax increase sooner and to higher levels following exposure of control cells to SM versus the CaM depleted cells. The decreased levels of Bax and the rapid increase in the levels of Bcl-2 and Bcl-xL are consistent with the CaM-depleted cells' resistance to SM-induced apoptosis.

2.4 Discussion

Last year we determined the time course of activation of "upstream" caspases 8, 9, and 10, and the "executioner" caspases 3, 6, and 7. The upstream caspases -8 and -9 were found to be both activated in a time-dependent fashion, although caspase-8 was cleaved prior to caspase-9 (0.5 h vs. 2 h), and little cleavage of caspase-10 was observed. Activation of caspase-8 is consistent with a Fas-mediated pathway of apoptosis, while activation of caspase-9 is consistent with a mitochondrial pathway of apoptosis. These results are consistent with the activation of both death receptor and mitochondrial pathways by SM. In addition, the activation of caspase-9 is inhibited using CaM antisense. Since the ratio of pro and antiapoptotic members of the Bcl-2 family determine the stability of the mitochondria, and subsequent release of cytochrome c and caspase-9 activation, we determined the time course and dose response for their activation. By performing both time course and dose response experiments, we found that depletion of CaM by antisense RNA maintained higher levels of Bcl-2 and Bcl-xL in HFK following exposure to SM. In addition levels of Bax were reduced in the CaM-depleted cells compared to controls following exposure to SM. Thus, depletion of CaM by antisense RNA resulted in the stabilization of mitochondria and inhibited the activation of caspase-9. This finding has direct implications for the utilization of CaM inhibitors to reduce SM toxicity as described in the next section.

3. USE OF CALMODULIN INHIBITORS TO BLOCK BAD PHOSPHORYLATION

3.1 Introduction

Bcl-2 is an anti-apoptotic protein located on the nuclear membrane, ER, and outer mitochondrial membrane. Several Bcl-2-related proteins have been described, including Bcl-xL, and Bcl-w, both of which are anti-apoptotic. In addition, some Bcl-2-related proteins are anti-apoptotic, including Bax, Bak, and Bad. In numerous recent studies, Bad has been implicated as a key player in programmed cell death (Datta et al., 1997; del Peso et al., 1997; Hsu et al., 1997; Scheid and Duronio, 1998; Yang et al., 1995; Zha et al., 1997; Zundel and Giaccia, 1998) and the Ca2+ /CaM-regulated protein, calcineurin, has been shown to interact with the Bcl-2 family members (Shibasaki et al., 1997), and to dephosphorylate Bad (Wang et al., 1997). This dephosphorylated form of Bad can interact with Bcl-2 or Bcl-XL and induce apoptosis (Zha et al., 1997).

To determine if this process can be blocked using specific inhibitors of CaM, we utilized the CaM-specific inhibitor W-13, along with its structurally similar analogue, W-12, which does not bind to CaM, as a control.

3.2 Materials and Methods

a. ***Culture of primary and immortalized human keratinocytes, and exposure to SM.***

Cells. Primary human foreskin keratinocytes (**HFK**) are prepared from human foreskin keratinocytes as described previously, and maintained in serum-free Keratinocyte Serum-Free Medium (SFM) supplemented with bovine pituitary extract and epidermal growth factor (EGF). Cells are grown to 80% confluency and split 1:5. NHEK in 75 cm^2 tissue culture flasks to 60-80% confluency, then exposed to HD diluted in KGM. Media is not changed for the duration of the experiments

b. Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, the blots were incubated with monoclonal or polyclonal antibodies (above) and then detected with appropriate peroxidase-labeled secondary antibodies (1:3000 dilution) and enhanced chemiluminescence (ECL, Amersham). Immunoblots were sequentially stripped by incubation for 30 min at 50 °C with a solution containing 100 mM 2-mercaptoethanol, 2%

SDS, and 62.5 mM Tris-HCl (pH 6.7), blocked again, and reprobed with additional antibodies to accurately compare different proteins from the same filter. Typically, a filter could be reprobed three times before there was detectable loss of protein from the membrane, which was monitored by Ponceau S staining after stripping.

3.3 Results

Examination of changes in the phosphorylation of the pro-apoptotic Bcl-2 family protein, Bad, to examine its role in Ca2+/CaM mediated pathways.

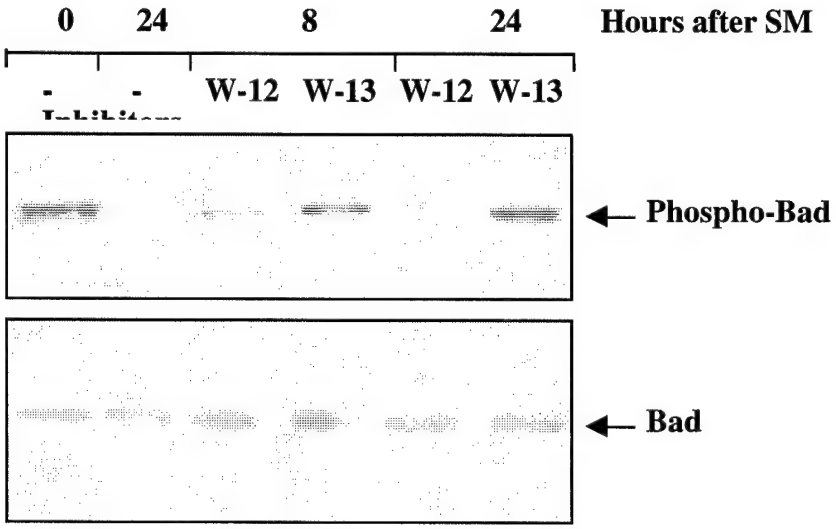


Fig. 4

When keratinocytes were treated with SM, there was a decrease in the phosphorylation level of Bad, as determined by specific antibody against phospho-Bad (Fig. 4). This dephosphorylation occurred as early as 8 h after SM exposure, and was complete by 24 h. In the presence of the specific CaM inhibitor, W-13, this dephosphorylation was inhibited. On the other hand, the total levels of Bad were fairly constant for all times after SM exposure and all inhibitor treatments (Fig. 4 bottom panel). Thus, the phosphorylation status of Bad appears to be more important for the induction of apoptosis than the absolute levels.

3.4 Discussion

Our model system has demonstrated a role for calmodulin in SM toxicity. In addition, we have also show the validity of the use of chemical inhibitors of CaM to block this response. While the absolute levels Bcl-2, Bcl-xL, and Bax are altered, the phosphorylation state of Bad is altered following SM exposure. Therefore, one mechanism for the action of CaM inhibitors is to block the dephosphorylation of Bad, preventing it from complexing with and inactivating Bcl-2, and thereby blocking activation of caspase-9 and cell death.

4. TIME COURSE AND DOSE RESPONSE OF DIFFERENTIATION PROTEINS DURING SM-INDUCED APOPTOSIS IN CONTROL, CALMODULIN-DEPLETED CELLS

4.1 Introduction

Examination of the role of CaM in altering other proteins that control terminal differentiation and apoptosis. Calmodulin plays pleiotropic roles in cell signaling. Last year we showed that SM induced changes in CaM, and furthermore, the differentiation-specific keratin K1 was also induced at the level of transcription. In the current year, we have extended these studies to examine the expression of involucrin, which is crosslinked in the mature squame of the epidermis.

4.2 Materials and Methods

Cam depletion, exposure to SM and immunoblot analysis were performed as described in previous sections.

4.3 Results

A dose response was first established. HFK were treated with either 0. 100, 200, or 300 μM SM, and immunoblot analysis was performed. Fig. 5 shows that in the presence of 100 μM SM a smear can clearly be detected which is the result of involucrin being crosslinked in the mature cornified envelope. However, in the presence of CaM antisense, this smear is not observed, indicating that SM-induced differentiation and involucrin

cross-linking is CaM dependent. We then performed a time course as shown in Fig 5, bottom panel. Involucrin is strongly crosslinked within 16 h in control keratinocytes. However in CaM-depleted cells, involucrin cross-linking is delayed.

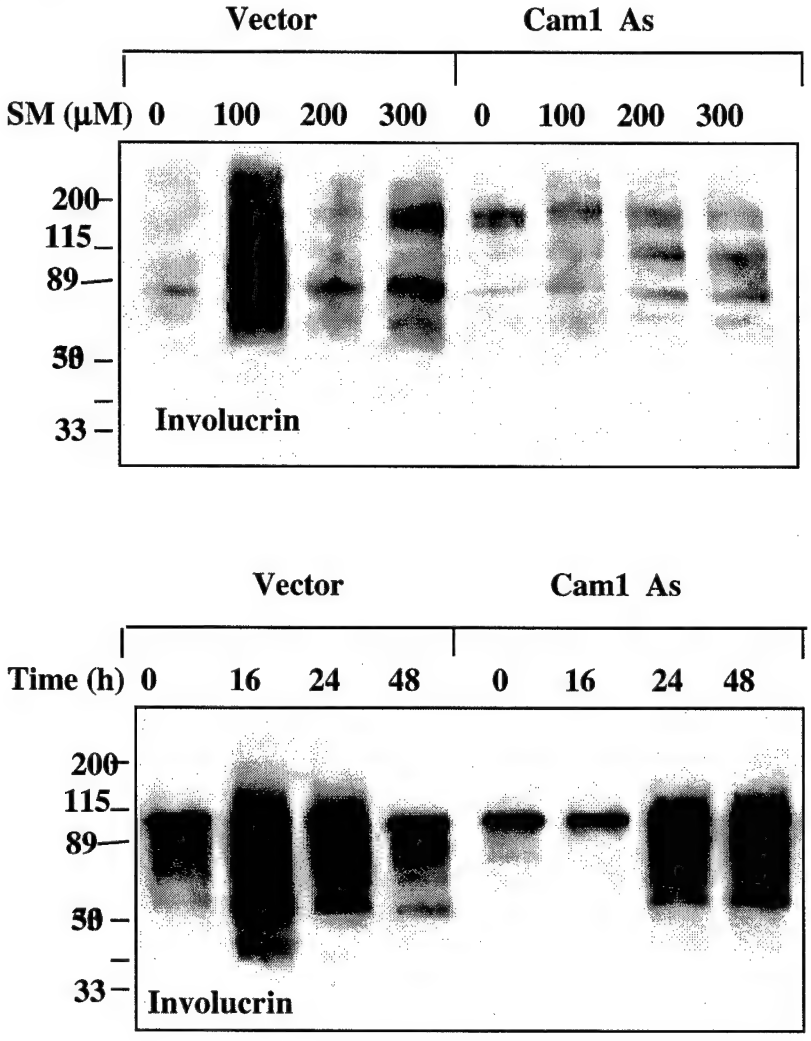


Fig. 5

4.4 Discussion

SM induces both differentiation and apoptosis in keratinocytes. This aberrant and rapid differentiation may result in the detachment of keratinocytes from the basal lamina and subsequent vesication response. The crosslinking of involucrin indicates that the terminal differentiation occurs very rapidly in the basal keratinocytes of cell culture, and probably in vivo as well. The depletion of CaM slows this process, and thus CaM inhibitors may be useful in the prevention of vesication as discussed in section 3.

5. THE ROLE OF FAS IN SM-INDUCED APOPTOSIS

5.1 Introduction

As outlined in the overview, many agents which damage DNA and induce apoptosis exert their effects by directly or indirectly activating death receptors. Last year, we demonstrated that Fas and Fas L are both upregulated following exposure to SM. Furthermore, caspase-8 is activated within 2 h after exposure of HFK to SM. To further analyze the importance of the death receptor pathway for SM toxicity, we utilized a dominant negative inhibitor of FADD (FADD-DN). In cells expressing FADD-DN, the recruitment of FADD to the death receptor complex is inhibited. Therefore, if FADD-DN blocks apoptosis, it can be concluded that the agent activates a death receptor pathway.

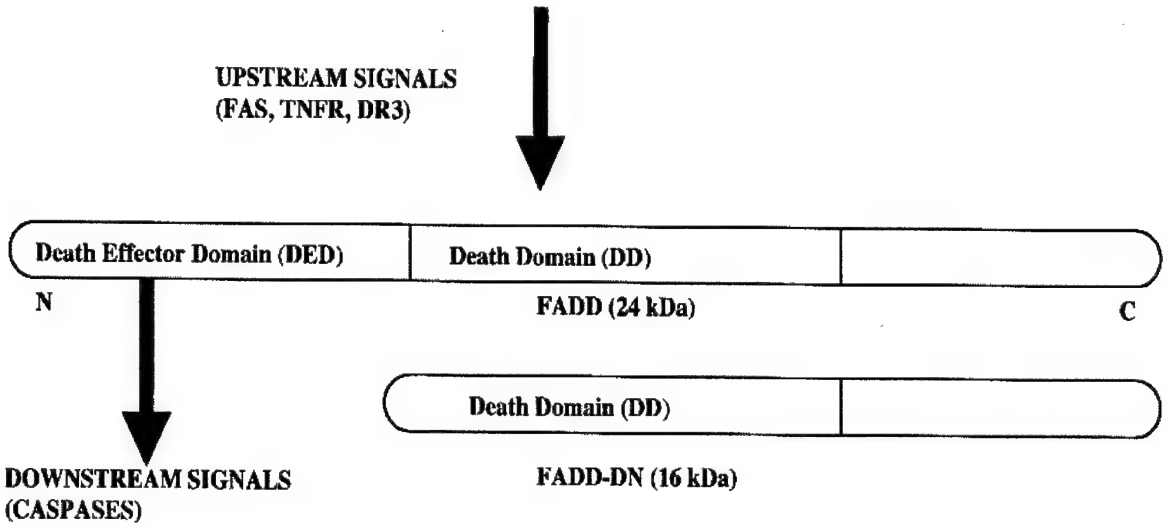


FIG. 6

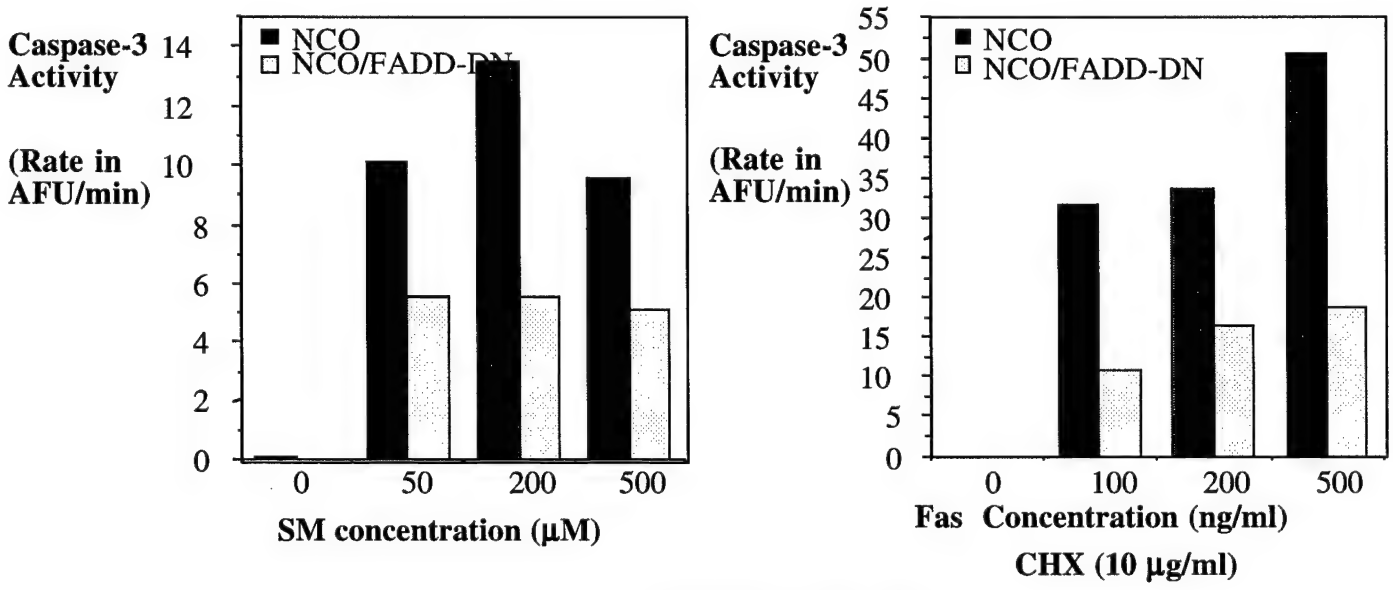
5.2 Materials and Methods

a. Plasmid. The FADD-DN plasmid was constructed to express a truncated FADD protein, as shown above in pcDNA 3.1 (Invitrogen). The expressed protein lacked the N terminal domain, responsible for recruiting and activating caspase-8 at the death receptor complex.

b. Transfection. Nco cells, derived from human foreskin keratinocytes (HFK) were transfected with empty vector or with FADD-DN using Lipofectamine (BRL), and stable clones were selected in the presence of G418, and maintained in serum-free Keratinocyte Serum-Free Medium (SFM) supplemented with bovine pituitary extract and epidermal growth factor (EGF). Cells are grown to 80% confluency and split 1:5. NHEK in

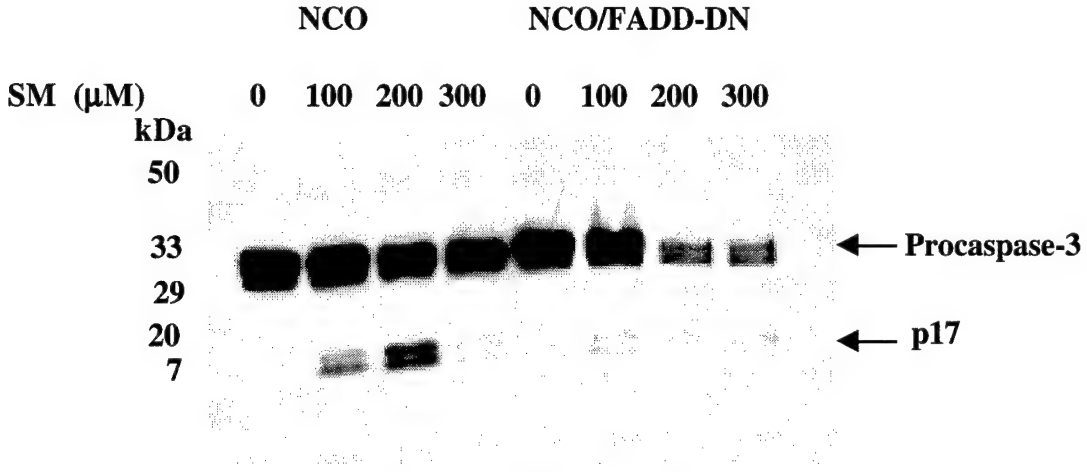
75 cm^2 tissue culture flasks to 60-80% confluency, then exposed to HD diluted in KGM. Media is not changed for the duration of the experiments

c. Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, the blots were incubated with monoclonal or polyclonal antibodies and then detected with appropriate peroxidase-labeled secondary antibodies (1:3000 dilution) and enhanced chemiluminescence (ECL, Amersham). Immunoblots were sequentially stripped by incubation for 30 min at 50 °C with a solution containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7), blocked again, and reprobed with additional antibodies to accurately compare different proteins from the same filter. Typically, a filter could be reprobed three times before there was detectable loss of protein from the membrane, which was monitored by Ponceau S staining after stripping.



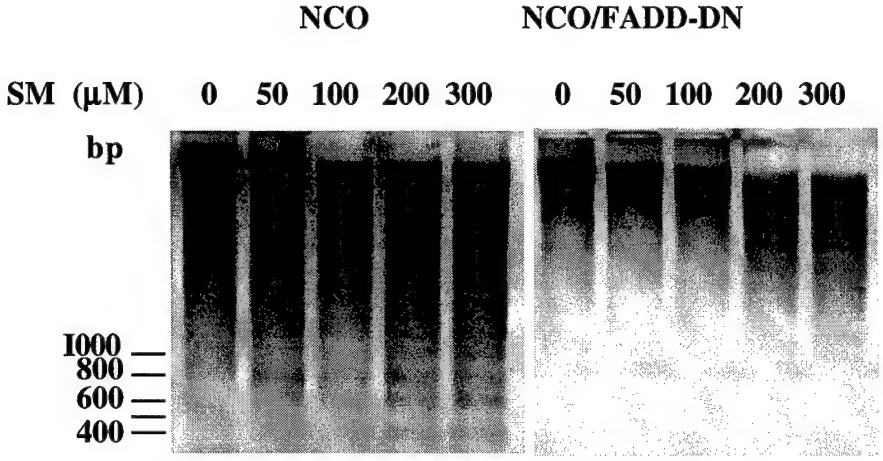
Caspase-3 Activation

Fig. 7



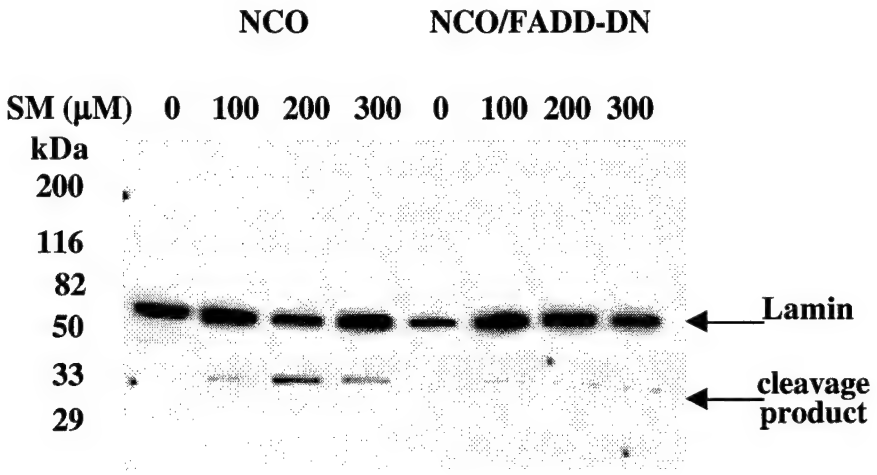
Caspase-3 Processing

Fig.8



Internucleosomal DNA fragmentation

Fig. 9



Caspase-6 mediated Lamin B1 cleavage

Fig. 10

5.3 Results

We first tested whether the FADD-DN construct could in fact suppress the death receptor pathway of apoptosis. Control keratinocytes (Nco), or cells stably expressing FADD-DN were treated with a Fas agonist antibody (clone CH11) to induce apoptosis. As our measure of apoptosis, we measured caspase-3 activity by fluorometric analysis, using DEVD-AMC as a substrate. Fig. 7 (right) shows that caspase-3 activity is suppressed in cells expressing the FADD-DN protein. Cells were then treated with either 0, 50, 200, or 500 μM SM, and extracts analyzed for caspase-3 activity. Fig. 7 (left) shows that caspase-3 activity is inhibited by FADD-DN.

Next, we analyzed whether expression of FADD-DN in keratinocytes could suppress the proteolytic processing of procaspase-3 into its active form. Fig. 8 shows that treatment of Nco keratinocytes with 100, 200, or 300 μM SM resulted in the processing of procaspase-3 into its active form. On the other hand this processing was almost completely suppressed in cells stably expressing FADD-DN.

A hallmark of apoptosis is the generation of multimers of nucleosome-sized DNA fragments as the result of the activation of apoptotic endonucleases which cleave the chromatin in the internucleosomal linker region. We therefore treated Nco or FADD-DN keratinocytes with increasing concentrations of SM, after which DNA was isolated and resolved on 1.5% agarose gels. Fig. 9 shows that a SM dose-dependent internucleosomal fragmentation is clearly visible in control Nco keratinocytes, but not in those expressing FADD-DN.

Another well established marker of apoptosis is the fragmentation of nuclei. This occurs partly because of the caspase-6 mediated cleavage of nuclear lamin at a specific sequence. We therefore analyzed the cleavage products of nuclear lamin following exposure to SM. Control Nco keratinocytes displayed a SM dose-dependent increase in the caspase-6 mediated cleavage of nuclear lamin (Fig. 10). On the other hand, this cleavage was almost completely suppressed in keratinocytes that stably expressed FADD-DN.

5.4 Discussion

In the past year, we demonstrated that SM toxicity is the result of differentiation, as well as induction of both death receptor and mitochondrial pathways of apoptosis. CaM mediates the mitochondrial apoptotic pathway, while the death receptor pathway is mediated by Fas and FADD. This is consistent with the results we obtained in the first year of the contract during which time we demonstrated that SM induced the activation of caspase-9, consistent with a mitochondrial pathway, as well as caspase-8, consistent with a death receptor pathway. In addition, our finding this year that involucrin is induced in a and crosslinked CaM-dependent fashion in response to SM is an extension of our finding that keratin K1 is induced by SM as well. These results will allow us to devise therapeutic measures that specifically target these pathways. In fact, we have already utilized the CaM-specific inhibitor, W-13 to block SM toxicity, and other CaM inhibitors have already been utilized clinically. In addition, there are specific inhibitors of the death receptor pathway of apoptosis that may be beneficial in reducing SM toxicity and vesication, including peptide inhibitors of specific caspases.

6. CONCLUSIONS

- **SM** in induces markers of terminal differentiation as well as apoptosis in HFK.

- When HFK are exposed to **SM** for 16 h, involucrin, a marker of terminal differentiation, is strongly induced at the protein level (as determined by immunoblot analysis) and cross-linked.

- When HFK are exposed to **SM**, levels of Bcl-2 and Bcl-xL are decreased, and Bax levels increase.

- Depletion of CaM by antisense RNA expression vectors prevents the SM-induced decreases in Bcl-2 and Bcl-xL, as well as the increase in Bax.

- The proapoptotic protein Bad is dephosphorylated within 8 h of SM exposure.

- SM-induced dephosphorylation of Bad is inhibited by the CaM inhibitor W-13.

- Fas and Fas L mediate many apoptotic responses. Blocking the death receptor complex by expression of a dominant negative FADD (FADD-DN) inhibits caspase-3 activation, processing, internucleosomal DNA cleavage, and caspase-6-mediated nuclear lamin cleavage.

Plans/Milestones for the Next Quarter

- Beginning of grafting experiments using FADD-DN and control keratinocytes
- Beginning of SM exposure experiments using animals with a deleted Fas gene

7. ACCOMPLISHMENT OF TASKS

C.2 Specific Aim I: Ca^{2+}-, CaM-, and Fas/TNF receptor-mediated changes in differentiation and apoptosis induced by **SM** in human epidermal keratinocytes will be further characterized in order to establish a molecular sequence of events following **SM** exposure.

Task 1: The sequence of events during **SM**-induced apoptosis were extensively characterized in the first year of the contract

Task 2: Time-course and dose-response experiments were performed to look for alterations in the differentiation protein involucrin as marker for **SM**-induced skin damage. Keratins were examined in the first year of the contract.

Task 3: Changes pro- and anti-apoptotic Bcl-2 family members have been examined in the presence and absence of CaM to determine their role in Ca^{2+} /CaM mediated pathways (**Task 3.3**). Levels of calmodulin, Fas, and Fas L were measured in the first year of the contract.

C.3 **Specific Aim 2**: Calmodulin and the Fas/TNF receptor pathway will be modulated in **SM**-exposed cultured epidermal keratinocytes by chemical inhibitors, antisense technology, neutralizing antibodies, and dominant-negative strategies

C.3.1
Task 1: The effects of modulation of CaM levels was determined by using antisense technology. It was determined that antisense constructs to CaM can alter the response of cultured epidermal keratinocytes to **SM** with respect to differentiation and apoptosis.

C.3.3
Task 3: The Fas-FADD pathway was altered in stable lines of transfected keratinocytes with FADD-DN. These are currently being grafted to nude mice for exposure to SM (C4.2 Task 2).

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